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The Growth of Simian Virus 40 (SV40) Host Range/Adenovirus Helper Function Mutants in an African Green Monkey Cell Line That Constitutively Expresses the SV40 Agnoprotein

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The simian virus 40 T-antigen carboxy-terminal mutants, *dl*A2459 and *dl*A2475, are cell line and temperature dependent for growth and plaque formation in monkey kidney cells. Although these mutants did form plaques on BSC-1 cells at 37°C, they were about fivefold less efficient for plaque formation than wild-type simian virus 40. These mutants did not grow in CV-1 cells and did not synthesize agnoprotein in those cells. CV-1 cells which constitutively express the agnoprotein were permissive for mutant plaque formation. However, late mRNAs, virion proteins, and progeny virion yields did not accumulate to wild-type levels during mutant infection of the agnoprotein-producing cells.

Expression of the simian virus 40 (SV40) large tumor (T) anitgen is essential throughout the infection cycle in permissive monkey kidney cells (for a review, see reference 31). T antigen is required for viral DNA replication (6, 18, 26, 28), autoregulation of SV40 early transcription (1, 29), and transactivation of the SV40 late promoter (3, 15, 16). Among the biochemical activities identified with T antigen are ATPase (7, 12), helicase (25), and specific binding to the SV40 origin of DNA replication (14, 22, 30). These activities have been mapped to regions of the T-antigen molecule extending from approximately amino acid 130 to 600 and are all required on the same T-antigen monomer for viral DNA replication (10, 32).

We have shown that the extreme carboxy terminus of SV40 T antigen mediates the host range/adenovirus helper function (*hr/hf*) (9, 24). Human adenoviruses produce very low progeny yields in most monkey kidney cell lines, but the block to productive infection of monkey cells can be overcome by SV40 T antigen (for a review, see reference 17). The adenovirus helper function activity of T antigen is contained within the carboxy-terminal 34 amino acids of T antigen (8, 21). Deletion mutants *dl*A2459 and *dl*A2475, which lack the carboxy-terminal 35 and 28 amino acids, respectively, of T antigen, were absolutely defective in providing helper function to human adenovirus in CV-1 cells, an African green monkey kidney cell line. These mutants also produced extremely low SV40 viral yields and were unable to form plaques in CV-1 cells at 37°C. The *hr/hf* mutants were more cold sensitive than wild-type SV40 for plaque formation and progeny yield in all cell lines tested. *dl*A2459 and *dl*A2475 were able to form plaques and produced higher viral yields on BSC-1 and Vero African green monkey kidney cells. Interestingly, human adenoviruses also grow productively in Vero cells, but not in other monkey kidney cell lines (11). These observations suggest that the adenovirus helper function of T antigen is also required by SV40 for productive infection in CV-1 cells. Complementation analysis has shown that the *hr/hf* mutants can form plaques in CV-1 cells when the carboxy terminus of T antigen is provided in *trans*, even when that region of T antigen is fused to the SV40 capsid protein, VP1 (34). The *hr/hf* activity of T antigen is, therefore, separable from the rest of T antigen.

Although Vero cells were the most permissive for the growth of SV40 *hr/hf* mutants, these cells do not survive well under plaque assay conditions. BSC-1 cells, which were more permissive for *hr/hf* mutant viral progeny production than CV-1 cells, were routinely used in plaque assays to determine titers of mutant viral stocks and measure mutant viral yields. Plaque sizes are relatively insensitive to the amount of infectious progeny produced within a single cell; for example, plaque size and rate of plaque enlargement are very similar for yields in the range of 500 to 5,000 PFU per cell. With smaller burst sizes, plaques will be smaller and enlarge more slowly, and at still lower burst sizes, plaques will not form at all. In addition, there probably exists a narrow range of burst sizes which result in plaque formation in only a fraction of infected cells. Since *hr/hf* mutant yields were both cell line and temperature dependent, and since plaques formed by these mutants in BSC-1 cells were smaller than wild-type plaques, it was possible that not all BSC-1 cells infected by the *hr/hf* mutants would give rise to a plaque. If this were the case, measuring mutant viral yields and virus stock titers by plaque assay would underestimate their true values.

We therefore performed an infectious-center assay. Confluent monolayers of BSC-1 cells were infected with wild-type SV40 or *dl*A2475 at a multiplicity of infection of 5 to infect all of the cells. Eighteen hours later, the infected cells were removed from the plates, counted, and serially diluted into suspensions of uninfected BSC-1 cells. The mixed cell suspensions were replated, incubated in cell culture medium for 4 h to settle the cells, and then overlaid with medium containing agar, as for plaque assays (32). After 8 days, duplicate samples were stained with neutral red and plaques were counted.

The results of this infectious-center assay are shown in Table 1. BSC-1 cells infected with wild-type SV40 produced plaques with 80 to 100% efficiency, while only 15 to 20% of *dl*A2475-infected cells resulted in visible plaques. Under conditions where the wild-type virus appeared least efficient,

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TABLE 1. Comparison of the ability of wild-type SV40 and *dl*A2475 to produce plaques on BSC-1 cells in an infectious-center assay

Infecting virus (no. of infected cells plated)	No. of plaques produced[a]	Efficiency of plaque formation[b]
Wild type		
400	TMTC[c]	ND[d]
200	TMTC	ND
100	50,70	0.6
50	36,45	0.81
25	25,20	0.89
12.5	19,13	1.20
*dl*A2475		
400	55,55	0.14
200	22,28	0.13
100	25,15	0.20
50	13,10	0.23
25	3,5	0.16
12.5	2,2	0.16

[a] Maximum numbers of plaques formed on each of duplicate plates.
[b] Calculated by dividing the average number of plaques on each set of duplicate plates by the number of infected cells plated.
[c] TMTC, Too many to count.
[d] ND, Not determined.

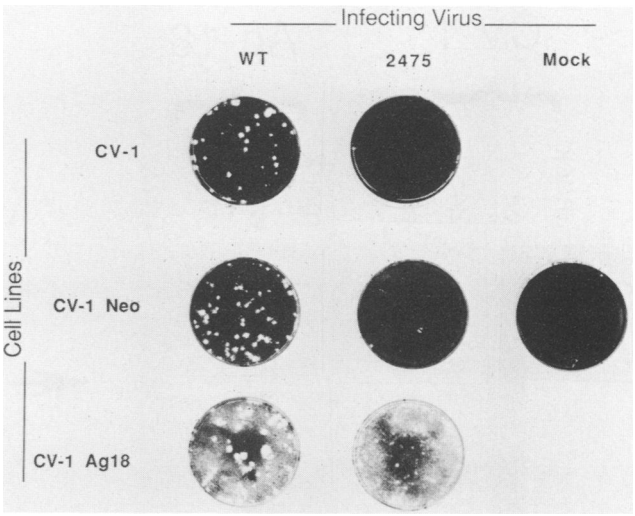

FIG. 1. Mutant and wild-type plaque formation on CV-1, CV-1 Neo, and CV-1 Ag18 cells. Cell monolayers were mock infected or infected with wild-type SV40 (wt) or *dl*A2475 as for plaque assays. Plaques were visualized and stained 10 days after infection.

e.g., at 100 infected cells plated, the actual efficiency was probably higher, since that many wild-type plaques would overlap on a 60-mm plate. The inefficiency of plaque formation after infection by mutant virus suggested that there is indeed a threshold in burst size, below which the number of surrounding cells that become infected during the plaque assay is insufficient to form a plaque before the death of the cell monolayer. The *hr/hf* mutant plaques on BSC-1 cells always appeared 2 or more days later and were significantly smaller and more difficult to detect than wild-type plaques. These observations also suggest a smaller burst size. Since only 15 to 20% of mutant-infected cells gave rise to a plaque in this infectious-center assay, titers of mutant virus stocks obtained by plaque assay on BSC-1 cells underestimated the true yield of infectious virions by a factor of 5 to 6.

Although the results obtained in this experiment could be explained by inefficient adsorption, penetration, or uncoating, we believe this is unlikely for the following reasons. Previous work has shown that similar amounts of viral DNA are made in mutant- and wild-type-infected cells (24, 34). This indicates that the events before DNA replication occur normally in mutant-infected cells, regardless of whether the cells support mutant plaque formation (BSC-1 cells) or not (CV-1 cells). We concluded that the block to productive infection in CV-1 cells involves late events in the lytic cycle (24, 33). Both late mRNA and the major viral capsid protein, VP1, accumulate to substantially lower levels in mutant infections of CV-1 cells than in wild-type SV40 infections. Also, the agnoprotein encoded in the SV40 late mRNA leader is detectable in wild-type infections of CV-1 cells but not in *hr/hf* mutant infections (24).

Mutant infection of CV-1 cells also results in an altered pattern of late mRNA start site usage. Primer extension data showed that the major late mRNA start site at SV40 nucleotide 325 was in fact the major site in wild-type-infected CV-1 cells. However, most late messages found in mutant infections initiate downstream from the agnoprotein translation initiation codon at nucleotide 335 and thus cannot encode the agnoprotein (24). The agnoprotein has not been detected in SV40 virions. It accumulates in SV40-infected cells at late times in the lytic cycle and is thought to play a role either in transport of VP1 to the nucleus or in virion assembly (2, 4, 20, 23). The agnoprotein is not essential to SV40 growth, because mutants which do not synthesize this protein have a small plaque phenotype but are still viable (13, 19, 27). Since the *hr/hf* mutants do not synthesize detectable levels of the agnoprotein, we investigated whether the agnoprotein supplied in *trans* could complement the SV40 growth defect in CV-1 cells caused by deletion of the carboxy terminus of T antigen.

The results of a plaque assay performed with wild-type SV40 and *dl*A2475 in the CV-1 Ag18 cell line, which are CV-1 cells that have been stably transformed with the DNA encoding the agnoprotein and which constitutively express this protein (5), are shown in Fig. 1. For comparison, the abilities of wild-type and mutant viruses to form plaques on parental CV-1 cells and on CV-1 cells transformed with the selectable marker gene for neomycin resistance (CV-1 Neo) are also shown. Confluent monolayers of each cell line were infected and cultured under standard plaque assay conditions at 37°C (32). When the maximum numbers of plaques were visible, the cells were fixed and stained with methylene blue for photography. Wild-type SV40 was able to form plaques equally well on all of the cell lines. The *hr/hf* mutant, however, was absolutely defective for plaque formation on the CV-1 and CV-1 Neo cells, even at dilutions as low as 10^{-3}. Plaques were detected on mutant-infected CV-1 Ag18 cells, indicating that the agnoprotein had a positive effect on the plaque-forming efficiency of the mutant.

We next examined whether the expression of the agnoprotein in CV-1 Ag18 cells allowed the *hr/hf* mutants to form plaques by increasing the levels of viral late message or capsid protein. RNase protection analysis of total cytoplasmic RNA from wild-type- and mutant-infected CV-1 and CV-1 Ag18 cells (Fig. 2) was performed as described previously (24). In this case, the probe used protects a 275-nucleotide fragment of SV40 late mRNA from RNase digestion. RNase-protected fragments were analyzed on a 6% polyacrylamide–50% urea gel. The intensity of the 275-base band, and therefore the amount of late mRNA, was not

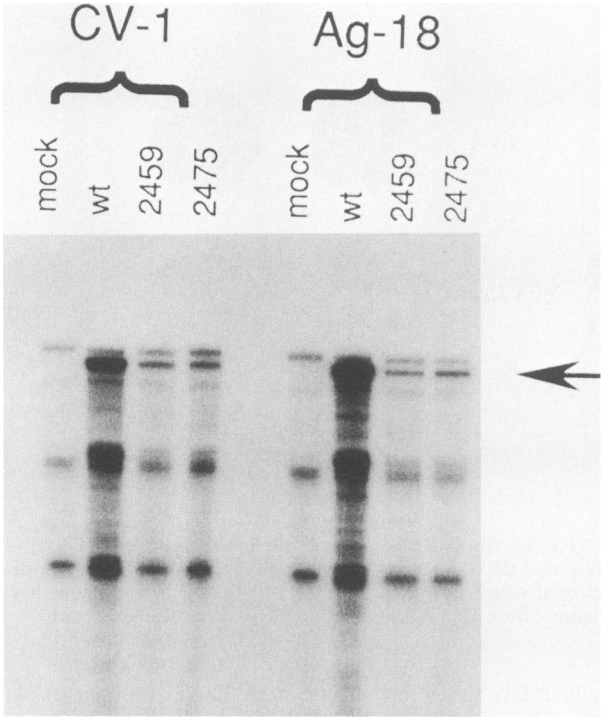

FIG. 2. RNase protection analysis of SV40 late mRNAs. Total cytoplasmic RNA was prepared from infected or mock-infected cultures, and equal amounts of RNA were annealed with ^{32}P-labeled anitsense RNA probe. The arrow indicates the position of the 275-nucleotide bands protected from RNase digestion by hybridization with SV40 late mRNAs. wt, Wild type.

increased in mutant (or wild-type) infections of CV-1 Ag18 cells relative to late mRNA levels in CV-1 cells. In all of the mutant-infected cells, the levels of late message were 5- to 10-fold lower than in wild-type-infected cells. We also mapped the 5′ ends of late viral mRNAs in mutant-infected CV-1 Ag18 cells and found the same non-wild-type pattern (data not shown) as reported previously for mutant-infected CV-1 cells (24). These results indicate that the presence of the agnoprotein had no effect on either the quantity or structure of SV40 late mRNA. Not surprisingly, the level of the major capsid protein, VP1, in mutant-infected CV-1 Ag18 cells was the same as in CV-1 cells, about fivefold lower than the level observed in wild-type SV40 infections (data not shown). We conclude that providing the agnoprotein in *trans* increased the plaque-forming efficiency of these mutants but did not affect the accumulation of viral late mRNA and protein.

The results of a viral yield assay comparing progeny production by wild-type SV40 and the *hr/hf* mutants in Vero cells, which were the most permissive cell line for mutant growth; in CV-1 cells, which were the least permissive; and in CV-1 Ag18 cells, which were able to support mutant plaque formation, are shown in Table 2. For this assay, multiple dishes of the three cell lines were infected with mutant or wild-type virus. Cultures were harvested on successive days, and maximum viral yields were determined by plaque titration on monolayers of BSC-1 cells. These data suggested that mutant progeny production was more efficient in CV-1 Ag18 cells than in CV-1 cells. In this experiment, *hr/hf* mutant yields were 250- to 800-fold lower than those of the wild type in CV-1 cells but only 10- to 20-fold lower in

TABLE 2. Comparison of wild-type SV40, *dl*A2459, and *dl*A2475 viral yields in Vero, CV-1, and CV-1 Ag18 cells

Cell line	Viral yield (PFU/cell) with:		
	Wild type	*dl*A2459	*dl*A2475
Vero	6,000	1,100	750
CV-1	788	3	<1
CV-1 Ag18	1,000	96	48

CV-1 Ag18 cells. For comparison, the mutant yields were about sixfold lower than wild-type yields in Vero cells. The infectious-center assay (Table 1) indicated that the mutant virus yields reported in Table 2 underestimated the actual yields of infectious mutant virus. However, it is clear from these data that the agnoprotein, which is expressed constitutively in CV-1 Ag18 cells, did not restore mutant progeny production to wild-type levels but that it did increase mutant virion production relative to that seen in the parental CV-1 cells.

Two factors probably account for the failure to increase mutant progeny yields in AG18 cells to wild-type levels. (i) The level and structure of viral late mRNAs produced in mutant-infected AG18 cells were unchanged from those seen in CV-1 cells. This leads to a reduction in VP1 levels in mutant-infected CV-1 or CV-1 AG18 cells to approximately 20% of the level seen in wild-type-infected cells. The absence of the agnoprotein is a result rather that the cause of the altered late mRNA structure. (ii) The level of agnoprotein synthesized in CV-1 AG18 cells was more than 100-fold lower than that seen in wild-type-infected CV-1 cells (S. Carswell, unpublished results). This small amount of agnoprotein may not be sufficient to maximize the yield of virions from the available capsid protein.

We have shown previously that deletion of the carboxy terminus of SV40 T antigen resulted in the loss of the adenovirus helper function, altered the host range of the mutants, and caused defects in viral late gene expression in CV-1 cells (9, 24, 33). Among these defects in viral late gene expression was the failure of the mutants to synthesize detectable levels of the agnoprotein in CV-1 cells. We have shown here that constitutive expression of the agnoprotein in CV-1 cells allowed the *hr/hf* mutants to form plaques in those cells but did not affect late mRNA or capsid protein accumulation and did not restore progeny yields to wild-type levels. If, as the results of the infectious center assay suggested, there is a minimum burst size required for plaque production, then it seems likely that providing the agnoprotein in *trans* was sufficient to boost the level of mutant virion production above that threshold. This interpretation is consistent with evidence that the agnoprotein plays a role in the transport of capsid protein to the nucleus or in the assembly and maturation of virions (2, 4, 23). It is also consistent with our observation that the levels of VP1 which accumulate in mutant-infected CV-1 cells were 5- to 10-fold lower than in wild type-infected cells, whereas the viral yields were several-hundred fold lower (24), suggesting inefficient production of progeny virions from the available capsid protein pools. We conclude from the studies described here that the failure to produce the agnoprotein in *hr/hf* mutant-infected cells is not directly responsible for the altered structure of viral late mRNAs in mutant-infected cells but rather is an indirect consequence of this alteration. Loss of the *hr/hf* function of T antigen affects viral yields indirectly. In mutant-infected cells, the level of viral late mRNAs is reduced, leading to reduced production of capsid proteins;

late mRNA structure is altered such that agnoprotein is not produced; and finally, in the absence of agnoprotein, virion assembly from available capsid proteins occurs with reduced efficiency.

The carboxy-terminal 35 amino acids have been shown to be critical for the *hr/hf* function (24, 33, 34). We described previously (34) SV40 mutants in which the C terminus of large T antigen was fused to VP1, and we demonstrated that one of these mutants, *dl*(inv)2408E, was able to complement *hr/hf* mutant *dl*A2459 for plaque formation on CV-1 cells. This raises the question of whether the carboxy-terminal 35 amino acids of large T fused to VP1 provide *hr/hf* activity or whether they complement *dl*A2459 by some other mechanism. Studies presented here and described previously by Stacy et al. (24) indicate that *hr/hf* mutations result in production of late mRNAs at a reduced level and with 5′ ends shifted primarily to sites downstream of the agnoprotein AUG. We think it unlikely that the VP1-T-antigen fusion protein of *dl*(inv)2408E would be able to restore mRNA levels and structures to those seen during wild-type infection. We imagine that T antigen, through its interactions with the SV40 origin region and with various transcription factors, affects the frequency of transcription initiation and the location of mRNA 5′ ends. Mutant T antigens lacking the normal carboxy terminus could have altered interactions with cellular factors, resulting in the late mRNA patterns seen. It is difficult to imagine how the VP1-T antigen fusion protein could play the same role in transcription initiation as wild-type T.

How then might *dl*(inv)2408E act to complement *dl*A2459? The studies described here provide some insight into this problem. In the absence of detectable levels of the agnoprotein, *dl*A2459 and *dl*A2475 are unable to form plaques on CV-1 monolayers (9). Providing the agnoprotein by using the AG18 cell line permitted plaque formation to occur (Fig. 1). Barkan et al. (2) have described missense mutations in VP1 which permit virion production to occur normally in the absence of the agnoprotein. One interesting possibility is that the VP1-T antigen fusion protein of *dl*(inv)2408E might function in a manner similar to that of the missense mutants in VP1 described by Barkan et al. (2); by this model, the fusion of T-antigen sequences to VP1 might relieve the requirement for the agnoprotein for proper transport to the nucleus and efficient assembly into progeny virions. If this were true, it would mean that the carboxy terminus of T antigen could perform two distinct functions during SV40 infection: the *hr/hf* function when it is attached to the remainder of T antigen, and, serendipitously, a function to relieve the agnoprotein requirement for efficient progeny virion formation. A major mystery which remains is how fusions between the carboxy terminus of T antigen and various adenovirus proteins act to permit efficient production of human adenovirus progeny in monkey cells (31). An understanding of the biochemical basis of the various activities of the carboxy terminus of T antigen will require further investigation.

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